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Dose-Dependent Incidence of Hepatic Tumors in Adult Mice following Perinatal Exposure to Bisphenol A

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Running Title: Hepatic Tumors in Mice Perinatally Exposed to BPA

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Abstract

Background: Bisphenol A (BPA) is a high production-volume chemical with hormone-like properties that has been implicated as a potential carcinogen. Early life exposure has been linked to increased risk for precancerous lesions in mammary and prostate glands and the uterus, but no prior study has shown a significant association between BPA exposure and cancer development.

Objective: We explored the effects of exposure to BPA during gestation and lactation on adult incidence of hepatic tumors in mice.

Methods: Isogenic mice were perinatally exposed to BPA through maternal diets containing one of four environmentally relevant doses (0, 50 ng, 50 μg, or 50 mg of BPA per kg diet) and approximately one male and one female per litter were followed until 10 months of age. Animals were tested for known risk factors for hepatocellular carcinoma, including bacterial and viral infections.

Results: We report dose-dependent incidence of hepatic tumors in exposed 10-month mice. 23% of offspring presented with hepatic tumors or preneoplastic lesions. A statistically significant dose-response relationship was observed, with an odds ratio for neoplastic and preneoplastic lesions of 7.23 (95% CI: 3.23, 16.17) for mice exposed to 50 mg BPA per kg diet compared with unexposed controls. Observed early disease onset, absence of bacterial or viral infection, and lack of characteristic sexual dimorphism in tumor incidence support a non-classical etiology.

Conclusions: To our knowledge, this is the first report of a statistically significant association between BPA exposure and frank tumors in any organ. Our results link early life exposure to BPA with the development of hepatic tumors in rodents, with potential implications for human health and disease.

Introduction

Bisphenol A (BPA) is an environmentally ubiquitous, high production-volume chemical that has been linked to cardiovascular, immune, neuroendocrine, and reproductive endpoints (Diamanti-Kandarakis et al. 2009). Biomonitoring studies routinely detect levels of BPA in urine in greater than 90% of adults in the United States, indicating that exposure to BPA is widespread (Calafat et al. 2008). BPA has been classified as an endocrine disruptor, and has been implicated in alterations in tissue enzyme and hormone receptor levels, interaction with hormone response systems, and cellular changes suggestive of carcinogenic potential (vom Saal et al. 2007).

The last large-scale evaluation of BPA's potential carcinogenicity in multiple target organs was a National Toxicology Program (NTP) 2-year chronic feed study conducted in 1982, which employed doses ranging from 1,000-10,000 ppm BPA. Results provided inconclusive evidence for BPA's carcinogenicity in the context of adult exposure. Non-significant incidences of liver tumors were reported in both sexes of rats and mice (National Toxicology Program (NTP) 1982). Subsequent early life BPA exposure studies that examined mammary (Vandenberg et al. 2007) and prostate (Prins et al. 2008) glands and the uterus (Bergeron et al. 1999) reported precancerous lesions following perinatal BPA exposure, but none have shown direct tumor development. Thus far, research on BPA and cancer has focused on reproductive estrogen-target organs, despite the ability of non-reproductive organs, such as the liver, to express estrogen receptors and respond to steroid hormone signaling (Cui et al. 2013) . Here we evaluate the effects of perinatal exposure to BPA at three environmentally relevant levels and show dose-dependent incidence of hepatic tumors in adult mice at 10 months of age. To our knowledge, this is the first statistically significant report of frank tumors, in addition to precancerous lesions, in any organ following perinatal or adult BPA exposure. Classically, both male humans and rodents

are two to four times as likely to develop hepatocellular carcinoma (HCC) as compared to females (Hoenerhoff et al. 2011). Liver tumors are uncommon in rodents prior to 12 months of age and often present at or later than 20 months (Maronpot 2009). The combination of observed early disease onset and lack of characteristic sexual dimorphism in tumor incidence support a non-classical etiology.

Methods

Animals and diets

Mice were obtained from a colony that has been maintained with sibling mating and forced heterozygosity for the viable yellow *Agouti* (A^{vy}) allele, resulting in a genetically invariant background (Waterland and Jirtle 2003). The A^{vy} mutation initially arose spontaneously in C3H/HeJ mice; animals carrying the mutation were backcrossed with C57BL/6J mice, followed by > 220 generations of sibling mating. Based on these crosses, animals are calculated to be genetically 6.25%-25% C3H/HeJ and 75%-93.75% C57BL/6J (Waterland and Jirtle 2003). The reported rate of spontaneous or induced hepatocellular carcinoma in C57BL/6J mice is variably reported as 2%-10%. The C57BL/6J strain as been classified in numerous publications as "relatively resistant" to hepatocellular carcinoma (Maronpot 2009). The incidence rate observed in our control animals is consistent with the reported rate in C57BL/6J mice.

Virgin wild-type *a/a* dams were randomly assigned to phytoestrogen-free AIN-93G diets (diet 95092, with 7% corn oil substituted for 7% soybean oil) supplemented with one of four doses of BPA (0, 50 ng, 50 µg, or 50 mg BPA per kg diet). All diet ingredients were supplied by Harlan Teklad, except BPA, which was supplied by the National Toxicology Program (NTP, Durham, NC). Diet composition is available online at www.harlan.com.

Wild-type (*a/a*), 6-week-old, virgin dams were exposed to their assigned BPA diets for two weeks prior to mating and housed in polycarbonate-free cages with *ad libitum* access to diet and BPA-free water. At eight weeks of age, virgin dams were mated once to A^{vy}/a sires and were impregnated within 0.5 to 5 days following co-housing with males. Sires were briefly exposed to diets containing BPA during the mating period (0.5 to 5 days). Pups were housed with their respective dams and fed their respective BPA diets until weaning at postnatal day 22. Pups were then housed with a same-sex A^{vy}/a sibling on standard phytoestrogen-free control diet until 10 months of age (Anderson et al. 2012; Anderson et al. 2013).

This mating scheme produces ~ 50% wild-type (*a/a*) offspring and ~ 50% heterozygous (A^{vy}/a) offspring. For this study, a subset of wild-type animals, approximately 1 male and 1 female per litter, was followed until 10 months of age: control diet (n = 19 offspring; n = 10 males and n = 9 females), 50 ng BPA/kg diet (n = 20 offspring; n = 10 males and n = 10 females), 50 µg BPA/kg diet (n = 21 offspring, n = 10 males and n = 11 females), or 50 mg BPA/kg diet (n = 18 offspring, n = 9 males and n = 9 females). This subset of offspring mice was assessed for metabolic and activity outcomes (Anderson et al. 2013).

Associated estimates of daily BPA exposure levels, based on a dam weighing 25 g consuming 5 g chow daily, are 0, 10 ng BPA/kg body weight/day, 10 µg BPA/kg body weight/day, and 10 mg BPA/kg body weight/day, respectively. These diets were chosen to capture both mean and maximum human environmental exposures to BPA, reported recently to range from 0.1-5 µg /kg body weight/day (Vandenberg et al., 2013). BPA exposure within human relevant ranges was confirmed with direct measurements in liver tissue of a subset of exposed and control animals (Anderson et al. 2012). For example, total liver BPA measurements in animals fed the highest dose of 50 mg BPA /kg chow ranged from 9.5-870 µg BPA /kg liver, which captures the

maximum human environmental exposure indicated by human fetal liver measurements ranging from below the limit of detection to 96.8 μg BPA /kg liver (Anderson et al. 2012). Livers from mice fed diets containing 50 μg BPA /kg chow and 50 ng BPA /kg chow exhibited < LOQ-11.3 μg BPA /kg liver (mean 2 μg /kg; median 0.6 μg /kg) and < LOQ-13 μg BPA /kg liver (mean 2.8 μg /kg; median 0.3 μg/kg), respectively (Anderson et al. 2012). To prevent possible BPA contamination, animals were singly housed in polypropylene cages, no polycarbonate plastics were used in animal management, and animal drinking water was tested once prior to the beginning of the exposure study by an independent, accredited public health and safety organization (NSF International, Ann Arbor, Michigan, www.nsf.org). These mice were housed in an AAALAC –approved facility with a 12-hr light cycle, ~50% relative humidity, 72 ± 2°C. Animals used in this study were maintained in accordance with the Institute of Laboratory Animal Resources guidelines (ILAR 1996) and were treated humanely and with regard for alleviation of suffering. The study protocol was approved by the University of Michigan Committee on Use and Care of Animals.

Histopathologic evaluation

Upon dissection at 10 months of age, liver tissue was flash frozen in liquid nitrogen and later formalin fixed and paraffin embedded; for each mouse, 2-3 slides containing liver sections, both with and without grossly visible masses, were evaluated for histopathology. Liver lesions were classified by light microscopy by an exposure-blinded, board-certified veterinary pathologist (ILB), according to recently revised, standardized guidelines established by the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project (Thoolen et al. 2010). This project represents consensus criteria for histopathological lesions in rodents as established by the North American, European, British, and

Japanese Societies of Toxicologic Pathology. Hyperplastic nodules were not classified as "regenerative" or "non-regenerative," as specified in INHAND, because hepatopathic lesions, such as inflammation and oval cell hyperplasia, were present, but no significant markers of liver injury, such as necrosis or fibrosis were observed. Representative photomicrographs were taken using an Olympus DP72 12.5 megapixel digital camera mounted on an Olympus BX45 light microscope with manufacturer's software (DP2-BSW, Olympus). Photo processing and composite plate construction were performed in Adobe Photoshop CS4.

Bacterial and viral screens

A total of 8 animals from our colony (n = 4 10-month-old mice and n = 4 post-natal day 22 (PND22) mice) were tested by PCR for infection with *H. hepaticus* or *H. mastomyrinus* using previously published primers and positive controls obtained from Judith S. Opp in the laboratory of Vincent Young at University of Michigan, under published PCR conditions (Eaton et al. 2011). Four of the eight animals tested were 10-month animals included in the present study (one from each exposure group: two with hepatic tumors, one each from the medium and high dose groups; two without hepatic tumors, one each from the control and low dose groups.) The remaining four animals were siblings of the animals in the present study euthanized at PND22 (Anderson et al. 2012); one animal from each exposure group was tested. Testing was performed after the completion of the present study. We confirmed our animal selection and screening protocol with a board-certified veterinary pathologist (ILB).

Serology testing for murine hepatitis virus (MHV) was performed via enzyme-linked immunosorbent assay (ELISA) every 6-8 weeks on sentinel animals not included in the present study (3 animals per 50-70 experimental cages) housed in cages with small amounts of soiled

bedding from randomly sampled experimental cages, changed once weekly (Wunderlich et al. 2011). Sentinel animals were co-housed in our animal facilities during our exposure study.

SNP genotyping

DNA was isolated from spleens of three male animals 200 days old [one Avy/a, one wild-type a/a, which were provided by Dr. Jirtle (Duke University) and one C57BL/6J purchased from The Jackson Laboratory (stock #000664) as a 21-day old weanling]. These mice were maintained in an AAALAC –approved facility with a 12-hr light cycle, ~50% relative humidity, 72 ± 2°C, and fed Purina LabDiet 5001 in shoebox-style polycarbonate cages (27 cm x 15 cm x 13 cm) with corn cob bedding (Bed-O' Cobs ¼®, Maumee, OH). The DNA was purified using a standard protocol of phenol/chloroform extraction followed by ethanol precipitation and dissolved in water. The mice were genotyped by Geneseek (Neogen) on the Mega Mouse Universal Genotyping Array (MegaMUGA) for 74,800 microsatellite markers, spaced at ~33 KB intervals throughout the mouse genome. Data were processed in PLINK (v1.07, pngu.mgh.harvard.edu/~purcell/plink/) and SAS (v9.3, SAS Institute, Cary, NC). Genotypes are available from the Mouse Phenome Database, MPD:484 at http://phenome.jax.org.

Statistical analysis

Variables

We histologically identified 16 total tumors, benign and malignant, as well as four hyperplastic nodules, two of which co-occurred with tumors. Neoplastic and pre-neoplastic lesions were grouped in four different binary variables (present/absent): (1) malignant hepatocellular carcinomas (HCC) only (n = 13); (2) benign hepatic adenomas (HA) only (n = 3); (3) all tumors combined (HCC and HA; n = 16); and (4) combined tumors and hyperplastic nodules (n = 18). Additional hepatic lesions analyzed as binary variables included: steatosis, inflammation,

Kupffer cell hyperplasia, oval cell hyperplasia, multinucleated hepatocytes, hepatocyte hypertrophy, and lipofuscin deposition. Total hepatic lesions (including tumors and all additional lesions listed) were evaluated as summary scores (1 point for presence of each lesion, summed across lesions). As steatosis and inflammation may represent non-specific background lesions whose inclusion may mask a true association, each score was tested in three ways: (1) inclusion of all hepatic lesions; (2) inclusion of all hepatic lesions, except steatosis; and (3) inclusion of all hepatic lesions, except steatosis and inflammation. Associations between dose groups and hepatic lesions (9 variables) and summary scores (3 variables) were tested in the models below.

Models

To facilitate comparison of results in our data with those of the 1982 NTP carcinogenicity bioassay on BPA, a nearly identical statistical strategy was employed. A total of 15 associations were tested between BPA exposure level and hepatic lesion(s); all associations were tested with both exact tests and logistic regression models, to account for bias inherent in each method, for a total of 30 models. Fisher's exact tests and Cochran-Armitage exact tests of trend were used to detect associations between dose groups and hepatopathic lesions listed above, and trends in those lesions by dose, respectively. Fisher's exact tests and Cochran-Armitage exact tests of trend were were run using the PROC FREQ statement with the EXACT option in SAS v9.3. Exact tests allow for conservative estimation of association significance given small cell counts, as compared to potential overestimation of significance by Chi-squared tests of association; therefore, we stratified data by sex in exact test analyses only, to prevent exacerbation of this bias. Logistic regression models, adjusted for clustering of mice within litters using generalized estimating equations (GEE), were used to test the same associations and trends. Poisson regression models, adjusted for clustering by litter, were run on summary score variables.

Clustering prevents overestimation of association significance due to errant assumption of animal independence. Neither exact tests nor logistic regression models allow for simultaneous adjustment for small cell counts and litter; bias inherent in both methods tends to overestimate significance. Statistical significance was defined as p-value < 0.05 for all analyses. All statistical analyses were completed using SAS (v9.3, SAS Institute, Cary, NC).

Results

Histopathological evaluation

We exposed mice during gestation and lactation through maternal diets containing one of four environmentally relevant doses of BPA (0, 50 ng, 50 µg, or 50 mg of BPA per kg diet) and followed approximately one male and one female offspring per litter until 10 months of age (n = 19, n = 20, n = 21, and n = 18, respectively). Upon dissection, 23.08% (n = 18/78) of offspring presented with neoplastic lesions (hepatocellular carcinomas or hepatic adenomas) or pre-neoplastic lesion (hyperplastic nodules), with an odds ratio of 7.23 (95% CI: 3.23, 16.17; p=0.014) for the 50 mg group compared with controls, and a significant dose-response on both Cochran-Armitage exact (p = 0.014) and logistic regression (p= 0.022) tests of trend (Figures 1A-C and 3; Table 1; Supplemental Material, Tables S1 and S2). As murine hepatic adenomas and carcinomas are related pathologies (Hoenerhoff et al. 2011), and preneoplastic lesions, including hyperplastic nodules, are often included in risk calculations following short-term carcinogenicity studies (Allen et al. 2007), we grouped benign adenomas, malignant carcinomas, and hyperplastic nodules for analysis. Results remained significant when preneoplastic lesions were excluded from analysis (Figure 3; Table 1; Supplemental Material, Tables S1 and S2). Upon stratification by offspring sex, we report a significant linear dose-response in a combination of neoplastic and preneoplastic lesions in female animals (Figures 2A and 3D;

Supplemental Material, Table S1). The presence of a statistically significant dose-response in females but not in males does not necessarily indicate that the dose-responses were significantly different between males and females.

Almost half of animals presented with oval cell, or hepatobiliary stem cell, hyperplasia (43.95%, n = 34/78), with significant odds ratios for the two highest dose groups (50 µg OR=5.40; 95% CI: 3.26, 8.93, p=0.001; 50 mg OR = 2.67; 95% CI: 1.75, 4.06, p = 0.020) (Figure 1D; Table 1; Supplemental Material, Tables S1 and S2). Approximately one-third of animals presented with hepatocyte hypertrophy (32.05%, n = 25/78), with a significant OR for the highest dose group (50 mg OR = 5.66; 95% CI: 2.57, 12.50, p = 0.028) (Figure 1E; Table 1; Supplemental Material, Tables S1 and S2). Incidences of oval cell hyperplasia and hepatocyte hypertrophy were significantly associated with increasing dose (Figure 4A and 4C; Supplemental Material, Tables S1 and S2). Animals with neoplastic lesions were significantly more likely to co-present with oval cell hyperplasia, hepatocyte hypertrophy, and Kupffer cell hyperplasia, suggesting a proliferative response to perinatal BPA exposure (Supplemental Material, Tables S5 and S6). We observed multinucleated hepatocytes in 8 animals (10.26%, n = 8/78), primarily in males in low-dose groups, although the association with BPA exposure was not statistically significant (Figures 1F, 2B, and 4D; Table 1). Inflammation (50.00%, n = 39/78) and steatosis (50.00%, n = 39/78) may represent non-specific markers of liver damage with age, rather than markers of chemical toxicity, as these lesions were distributed fairly uniformly across doses and controls, without any apparent pattern (Figure 1E, Table 1). Notably, no evidence of liver injury, such as fibrosis or necrosis, was present, suggesting that the proliferative lesions noted were not a regenerative response to injury. When inflammation and steatosis were excluded from analysis, the total number of hepatic lesions increased with dose, indicating that hepatic lesions that were

significantly associated with perinatal BPA exposure co-presented in the same animals (Supplemental Material, Tables S3 and S4). Exposed dams did not present with any overt signs of obesity or other adverse health outcomes.

Bacterial and viral screens

In order to rule out known bacterial and viral disease risk factors, we performed a representative PCR screen for potential bacterial infection with *Helicobacter hepaticus* or *Helicobacter mastomyrinus* and assessed murine hepatitis viral load via serology measurements. All animals evaluated tested negative on all bacterial and viral screens.

SNP genotyping

The mouse strain used in these experiments was previously calculated to contain 6.25%-25% of the C3H/HeJ genome and 75%-93.75% of the C57BL/6J genome (Waterland and Jirtle, 2003). C3H/HeJ mice are prone to spontaneous hepatocellular neoplasms and C57BL/6J are relatively resistant (Maronpot 2009). Up to 85% of the greater susceptibility of the C3H mouse to hepatocellular carcinomas can be attributed to the *Hcs7* (*Hepatocarcinogenicity sensitivity 7*) locus, located on the distal arm of chromosome 1 (Bilger et al. 2004; Drinkwater 1994). In order to empirically confirm the overlap between our strain's genome and the C57BL/6J genome, we genotyped 74,830 SNPs in two male mice derived from our colony and one male C57BL/6J mouse purchased from The Jackson Laboratory. Our strain's genome differed from the C57BL/6J genome at 5,247 SNPs in total, and at only six of 5,416 SNPs on chromosome 1, indicating that our mice are genetically 93% C57BL/6J overall and > 99% C57BL/6J on chromosome 1 (Supplemental Material, Table S7). Thus, our strain is genetically C57BL/6J at the *Hcs7* locus and, therefore, likely relatively resistant to spontaneous hepatocellular carcinoma.

Discussion

Here, we report findings of dose-dependent incidence of hepatic tumors following perinatal exposure to BPA in an isogenic mouse model. Although mammary carcinomas have been reported in rodents following perinatal BPA exposure (Acevedo et al. 2013), the link was not statistically significant. To our knowledge, this is the first study to demonstrate a statistically significant relationship between BPA exposure and frank tumors of any reproductive or non-reproductive estrogen-target organ. These tumors may be classified as early onset disease, as liver tumors are uncommon in all laboratory mouse strains prior to 12 months of age and often present at or later than 20 months (Maronpot 2009). We did not note any apparent sexual dimorphism in disease incidence, except in control animals. Classically, both male humans and rodents are two to four times as likely to develop hepatocellular carcinoma as compared to females (Hoenerhoff et al. 2011). The combination of observed early disease onset and lack of characteristic sexual dimorphism in tumor incidence support a non-classical etiology. These findings appear to be a function of dose and/or exposure timing, as the adult rats and mice in the National Toxicology Program's 1982 carcinogenicity bioassay on BPA were exposed to doses estimated to be 20 times to 200 times higher than the doses employed in this study, but no significant increase in hepatic tumors was reported (NTP 1982).

Interestingly, we replicated the NTP study observation of dose-dependent multinucleated hepatocytes (NTP 1982); the association between this lesion and BPA exposure was not statistically significant in either study. These abnormal cells may be found in aged mice but appear at younger ages following xenoestrogen exposure and may be associated with increased hepatocyte proliferation (Hayashi et al. 2008; Scampini et al. 1993). These data represent increased ploidy in mice without visible liver masses. BPA has previously been shown to induce

meiotic aneuploidy in female mice (Hunt et al. 2003). Aneuploidy is the most common characteristic of solid tumors in humans (Kops et al. 2005). The presence of several proliferative lesions in exposed mice, including multinucleated hepatocytes and oval and Kupffer cell hyperplasia, in the absence of cellular necrosis or fibrosis, indicates an isolated proliferative response, and not a regenerative response following liver injury (Thoolen et al. 2010). Prior studies have noted a connection between BPA exposure and oxidative stress (Babu et al. 2013; Moon et al. 2012); perhaps an exposure-mediated increase in reactive oxygen species (ROS) led to a concomitant increase in cellular proliferation in exposed mice via ROS signaling (Goodson et al. 2011; Hassan et al. 2012).

Animals tested negative on all bacterial and viral screens for infectious agents known to be promoters of hepatocellular carcinoma in rodents. As previously reported, gestational BPA exposure in these animals did not significantly influence litter size, survival, genotypic ratio, or sex ratio in comparison to control offspring (Anderson et al. 2012). Obesity and diabetes are well-documented risk factors for hepatocellular carcinoma in both rodents and humans. However, at 9 months of age, the exposed offspring examined in this study, regardless of tumor presence, exhibited body weights and serum glucose and insulin measurements at or below levels found in control animals (Anderson et al. 2013).

Since rodents have a high capacity for hepatocellular proliferation in response to liver damage, non-genotoxic factors may or may not be relevant to human exposures, although recent work suggests that molecular gene expression profiles of hepatocellular carcinomas in B6C3F1 mice are similar to those of humans (Hoenerhoff et al. 2011). Hepatocellular carcinoma is the sixth most common malignancy and the third most common cause of cancer-related deaths globally. Mortality rates in the United States are increasing more rapidly than for any other leading cancer,

and age-adjusted incidence rates have doubled in the past thirty years, with an increase in early onset disease in both sexes (Shaw and Shah 2011).

Although the majority (80%) of hepatocellular carcinomas in humans can be attributed to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, further study of BPA's role as a potential risk factor is warranted. Historically, use of first-generation oral contraceptives containing high doses of estradiol has been associated with increased rates of hepatic neoplasms, particularly hepatic adenomas (Giannitrapani et al. 2006). Recent studies have indicated that endogenous sex hormone levels can increase rates of carcinogenic conversion in HBV+ individuals (Wu et al. 2010). Ramirez et al., demonstrated that female rats given daily subcutaneous injections of 50 or 500 µg BPA (equivalent to 2.5-6.25 mg/kg BW and 25-62.5 mg/kg BW, respectively) from PND1-10 experienced a loss of growth hormone-dependent sexual dimorphism in the liver's ability to metabolize toxicants (Ramirez et al. 2012). Moon et al., showed that intraperitoneal doses of 0.05-1.2 mg/kg BW/day administered to mice for five days induced hepatic mitochondrial dysfunction (Moon et al. 2012). An epidemiological study of 1455 adults, aged 18-74, demonstrated a statistical association between increased urinary BPA and clinically abnormal concentrations of liver enzymes gamma-glutamyltransferase and alkaline phosphatase (Lang et al. 2008). Further, Betancourt et al., found that exposing lactating female rats to 250 µg/kg BW/day (estimated exposure to offspring 2.5-25 ng BPA/kg BW/day) led to an increase in offspring susceptibility to subsequent chemical carcinogenesis (Betancourt et al. 2012).

Our study design has several notable strengths. We exposed mice to three doses that span several orders of magnitude, and the lower two doses are classified as 'low dose' by two well-accepted definitions: a dose not exceeding the threshold of the EPA's reference dose of 50 µg/kg BW/day;

and a dose within the range of observed human environmental exposure levels (Vandenberg et al. 2013). Our model was an inbred rodent strain that is well accepted and relatively resistant to hepatic tumor development. We exposed animals through the diet, currently accepted as a dominant route of exposure to BPA in humans (vom Saal et al. 2007). Animals were exposed during the perinatal period, capturing outcomes that may depend on exposure during critical developmental time points. Finally, we statistically clustered our data by litter, a method not used in many earlier BPA studies, which represents a significant criticism of and barrier to interpretation of prior studies.

A limitation of this study is the absence of direct maternal and fetal internal BPA dose measurements. However, comprehensive maternal and fetal measurements have been previously described. Zalko *et al.* determined that fetal free BPA levels peaked at approximately 4 ng/g 30 minutes following subcutaneous injection of pregnant CD-1 mice with 25 μg BPA/kg BW, indicating that fetuses were exposed to approximately 6.25% of the administered dose (Zalko et al. 2003). Sieli et al. demonstrated that bioavailability of BPA is higher in adult female C57BL/6J mice following dietary exposure (100 mg BPA-d_6/kg diet, similar to this study's maximum dose of 50 mg BPA/kg diet), as compared to oral bolus administration, despite less efficient absorption of BPA when ingested (Sieli et al. 2011). In addition, mice exposed via diet exhibited higher maximum serum BPA concentrations and greater temporal delay in reaching maximum serum BPA concentrations as compared to those receiving oral bolus, indicating sustained circulating concentrations of BPA following dietary exposure (Sieli et al. 2011).

Our animal model and exposure scheme were initially chosen to evaluate the effects of perinatal BPA exposure on the mouse epigenome (Anderson et al. 2012) and adult obesity risk (Anderson et al. 2013), rather than liver tumorigenesis. However, SNP genotyping performed in this study

confirms that our model is genetically similar to C57BL/6J mice at *Hcs7*, the locus reported to be associated with this strain's resistance to hepatocellular carcinoma; thus the mice evaluated in this study represent a conservative model for liver cancer development. The limitations of this model are similar to that of any animal model, in that no direct conclusions can be drawn from this study on human health risk, particularly as human populations are genetically diverse and our model is isogenic. The use of an isogenic model, however, also represents a study strength, in that we were able to detect statistically significant outcomes without potentially confounding effects of individual differences in genetic susceptibility.

Conclusions

The significance of this study may be summarized as follows: (1) to our knowledge, these data represent the first report of frank tumors in any organ following perinatal or adult BPA exposure; (2) these findings underscore the critical importance of exposure timing when evaluating adverse outcomes, particularly in light of non-significant liver tumor data in peripubertally exposed rodents in the noted 1982 NTP study; and (3) these results implicate perinatal exposure to an environmentally ubiquitous chemical in the development of hepatic tumors, with potential implications for human health and disease.

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Table 1. Frequency of hepatic lesions in mice exposed perinatally to BPA. Frequencies of hepatic lesions in mice exposed perinatally to a control diet or to one of three doses of BPA (50 ng/kg diet, 50 μg/kg diet, or 50 mg/kg diet), by dose and sex. All values are shown as percent (proportion).

Hepatic lesion	Dose (kg/diet)	Total animals	Males	Females
Hepatic adenoma	Total	3.85 (3/78)	5.00 (2/40)	2.63 (1/38)
	Control	0 (0/19)	0 (0/10)	0 (0/9)
	50 ng BPA	0 (0/20)	0 (0/10)	0 (0/10)
	50 μg BPA	0 (0/21)	0 (0/11)	0 (0/10)
	50 mg BPA	16.67 (3/18)	22.22 (2/9)	11.11 (1/9)
Hepatocellular carcinoma	Total	16.67 (13/78)	17.50 (7/40)	15.79 (6/38)
	Control	10.53 (2/19)	20.00 (2/10)	0 (0/9)
	50 ng BPA	15.00 (3/20)	14.29 (1/10)	22.22 (2/10)
	50 μg BPA	14.29 (3/21)	18.18 (2/11)	12.50 (1/10)
	50 mg BPA	27.78 (5/18)	22.22 (2/9)	33.33 (3/9)
Neoplastic lesions[a]	Total	20.51 (16/78)	22.50 (9/40)	18.42 (7/38)
	Control	10.53 (2/19)	20.00 (2/10)	0 (0/9)
	50 ng BPA	15.00 (3/20)	10.00 (1/10)	22.22 (2/10)
	50 μg BPA	14.29 (3/21)	18.18 (2/11)	10.00 (1/10)
	50 mg BPA	44.44 (8/18)	44.44 (4/9)	44.44 (4/9)
Neoplastic and preneoplastic lesions[b]	Total	23.08 (18/78)	25.00 (10/40)	21.05 (8/38)
	Control	10.53 (2/19)	20.00 (2/10)	0 (0/9)
	50 ng BPA	15.00 (3/20)	10.00 (1/10)	20.00 (2/10)
	50 μg BPA	23.81 (5/21)	27.27 (3/11)	20.00 (2/10)
	50 mg BPA	44.44(8/18)	44.44 (4/9)	44.44 (4/9)
Oval cell hyperplasia	Total	43.95 (34/78)	45.00 (18/40)	42.11 (16/38)
	Control	26.32 (5/19)	40.00 (4/10)	11.11 (1/9)
	50 ng BPA	30.00 (6/20)	20.00 (2/10)	40.00 (4/10)
	50 μg BPA	66.67 (14/21)	72.73 (8/11)	60.00 (6/10)
	50 mg BPA	50.00 (9/18)	44.44 (4/9)	55.56 (5/9)
Kupffer cell hyperplasia	Total	12.82 (10/78)	7.50 (3/40)	18.42 (7/38)
	Control	15.79 (3/19)	20.00 (2/10)	11.11 (1/9)
	50 ng BPA	15.00 (3/20)	0 (0/10)	30.00 (3/10)
	50 μg BPA	9.52 (2/21)	9.09 (1/11)	10.00 (1/10)
	50 mg BPA	11.11 (2/18)	0 (0/9)	22.22 (2/9)

Hepatic lesion	Dose (kg/diet)	Total animals	Males	Females
Multinucleated hepatocytes	Total	10.26 (8/78)	17.50 (7/40)	2.63 (1/38)
	Control	0 (0/19)	0 (0/10)	0 (0/9)
	50 ng BPA	20.00 (4/20)	30.00 (3/10)	10.00 (1/10)
	50 μg BPA	14.29 (3/21)	27.27 (3/11)	0 (0/10)
	50 mg BPA	5.56 (1/18)	11.11 (1/9)	0 (0/9)
Steatosis	Total	50.00 (39/78)	47.50 (19/40)	52.63 (20/38)
	Control	52.63 (10/19)	50.00 (5/10)	55.56 (5/9)
	50 ng BPA	45.00 (9/20)	40.00 (4/10)	50.00 (5/10)
	50 μg BPA	57.14 (12/21)	54.55 (6/11)	60.00 (6/10)
	50 mg BPA	44.44 (8/18)	44.44 (4/9)	44.44 (4/9)
Inflammation	Total	50.00 (39/78)	42.50 (17/40)	57.89 (22/38)
	Control	57.89 (11/19)	50.00 (5/10)	66.67 (6/9)
	50 ng BPA	45.00 (9/20)	30.00 (3/10)	60.00 (6/10)
	50 μg BPA	42.86 (9/21)	36.36 (4/11)	50.00 (5/10)
	50 mg BPA	55.56 (10/18)	55.56 (5/9)	55.56 (5/9)
Hepatocyte hypertrophy	Total	32.05 (25/78)	27.50 (11/40)	36.84 (14/38)
	Control	15.79 (3/19)	20.00 (2/10)	11.11 (1/9)
	50 ng BPA	30.00 (6/20)	20.00 (2/10)	40.00 (4/10)
	50 μg BPA	33.33 (7/21)	27.27 (3/11)	40.00 (4/10)
	50 mg BPA	50.00 (9/18)	44.44 (4/9)	55.56 (5/9)
Lipofuscin deposition	Total	16.67 (13/78)	7.50 (3/40)	26.32 (10/38)
	Control	5.26 (1/19)	0 (0/10)	11.11 (1/9)
	50 ng BPA	15.00 (3/20)	0 (0/10)	30.00 (3/10)
	50 μg BPA	23.81 (5/21)	18.18 (2/11)	30.00 (3/10)
	50 mg BPA	22.22 (4/18)	11.11 (1/9)	33.33 (3/9)

[a]Neoplastic lesions are defined as a combination of benign adenomas and malignant carcinomas. [b]Neoplastic and pre-neoplastic lesions include adenomas, carcinomas, and pre-neoplastic nodules.

Figure Legends

Figure 1. Representative photomicrographs of hepatic lesions in BPA-exposed mice. *(A)* Hepatocellular carcinoma in a female mouse exposed to 50 mg BPA/kg maternal diet. *(B)* Hepatic adenoma in a male mouse exposed to 50 mg BPA/kg maternal diet. Arrows indicate line of demarcation between neoplasm and compressed adjacent normal parenchyma. *(C)* Hyperplastic nodule in a female mouse exposed to 50 ng BPA/kg maternal diet. Arrow shows a bile duct as part of a portal triad within the lesion, indicating preservation of hepatic architecture. *(D)* Oval cell hyperplasia (arrowheads) and increased Kupffer cells within sinusoids (Kupffer cell hyperplasia) in a male mouse exposed to 50 ng BPA/kg diet. *(E)* Degenerative changes including lipofuscin accumulation (arrow), hepatocellular hypertrophy (arrowhead), and steatosis (asterisks) in a female mouse exposed to 50 ng BPA/kg diet. *(F)* Multinucleated hepatocytes (arrows) in a male mouse exposed to 50 mg BPA/kg diet. Hematoxylin and eosin. Original magnification x400. Bar 50 μm.

Figure 2. Mice exposed perinatally to BPA exhibit both linear and non-monotonic dose responses in a lesion-specific manner. *(A)* Mice exposed perinatally to BPA exhibit a statistically significant trend in a combination of neoplastic and preneoplastic hepatic lesions. *(B)* Mice exposed perinatally to BPA exhibit a non-monotonic trend in multinucleated hepatocytes, although this trend is not statistically significant. Gray line and hash symbol indicate total animals. Blue line, and pink line and hash symbol, indicate male and female animals only, respectively. *#p* for trend < 0.05 on Cochrane-Armitage exact test of trend and logistic regression (Supplemental Material, Table S2).

Figure 3. Dose-dependent incidence of hepatic tumors in mice exposed perinatally to BPA. *(A)* Mice perinatally exposed to BPA exhibit a statistically significant trend in hepatic adenomas (n = 3/78). *(B)* Mice perinatally exposed to BPA exhibit hepatocellular carcinomas (n = 13/78). *(C)* Mice perinatally exposed to BPA exhibit a statistically significant trend in neoplastic hepatic lesions (n = 16/78). *(D)* Mice perinatally exposed to BPA exhibit a statistically significant trend in neoplastic and preneoplastic hepatic lesions (n = 18/78). Gray bars indicate total animals. Blue and pink bars indicate male and female animals only, respectively. Spanning bar in 3A and upper spanning bars in 3C and 3D indicate trends in total animals. Lower spanning bars in 3C and 3D indicate trends in female animals. #*p* for trend < 0.05 on both Cochrane-Armitage exact test of trend and logistic regression, with the exception of exact test only for hepatic adenoma. ##*p* for trend < 0.1. *Odds ratio $p < 0.05$ on logistic regression.

Figure 4. Dose-dependent incidence of proliferative lesions in mice exposed perinatally to BPA. *(A)* Mice perinatally exposed to BPA exhibit a statistically significant trend in oval cell hyperplasia. *(B)* Mice perinatally exposed to BPA exhibit no clear trend in Kupffer cell hyperplasia. *(C)* Mice perinatally exposed to BPA exhibit a statistically significant trend in hepatocyte hypertrophy. *(D)* Mice perinatally exposed to BPA exhibit no clear trend in multinucleated hepatocytes. Gray bars indicate total animals. Blue and pink bars indicate male and female animals only, respectively. Upper spanning bars in 4A and 4C indicate total animals. Lower spanning bars in 4A and 4C indicate female animals. #*p* for trend < 0.05 on both Cochrane-Armitage exact test of trend and logistic regression. ##*p* for trend < 0.1. *Odds ratio $p < 0.05$ on logistic regression.

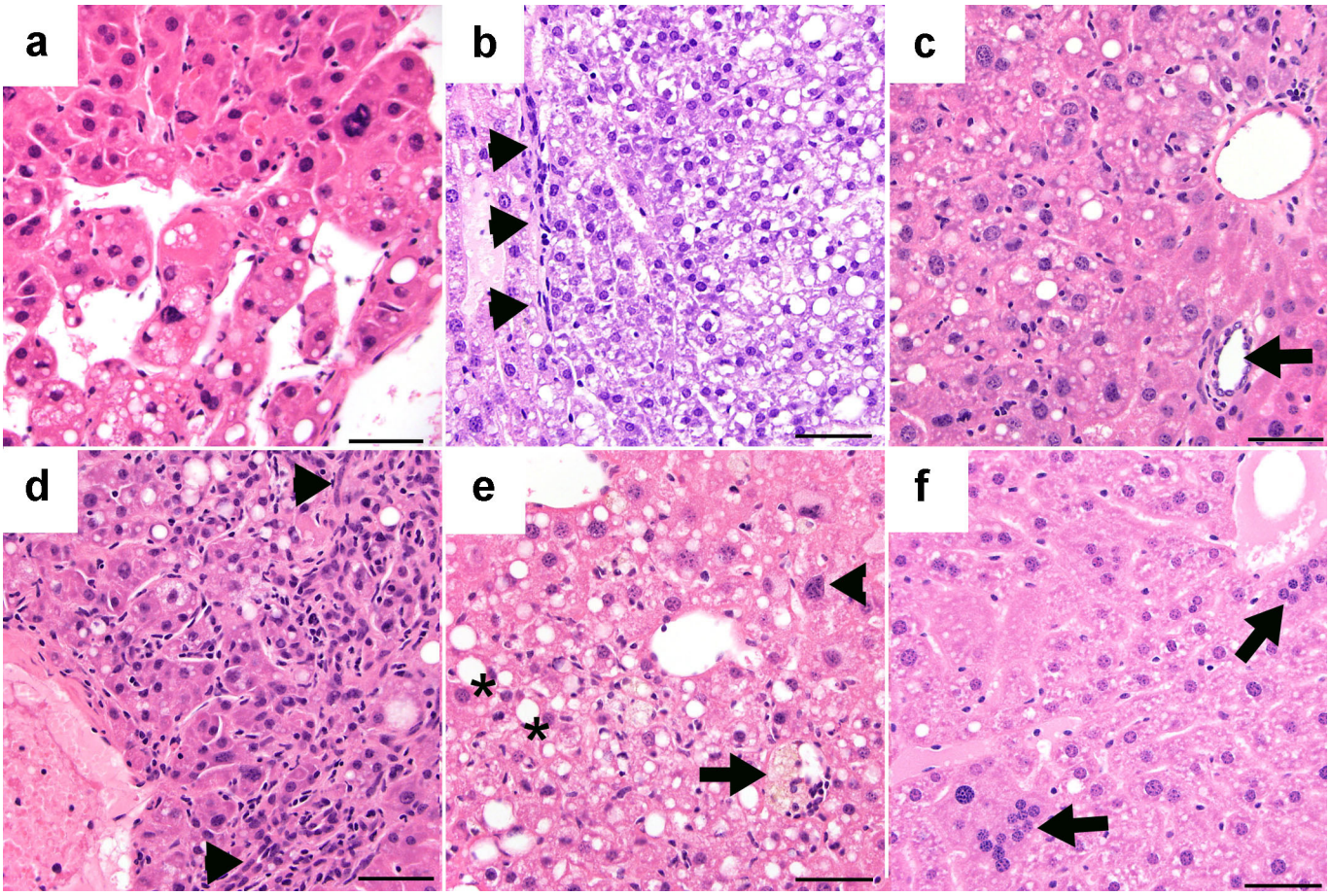
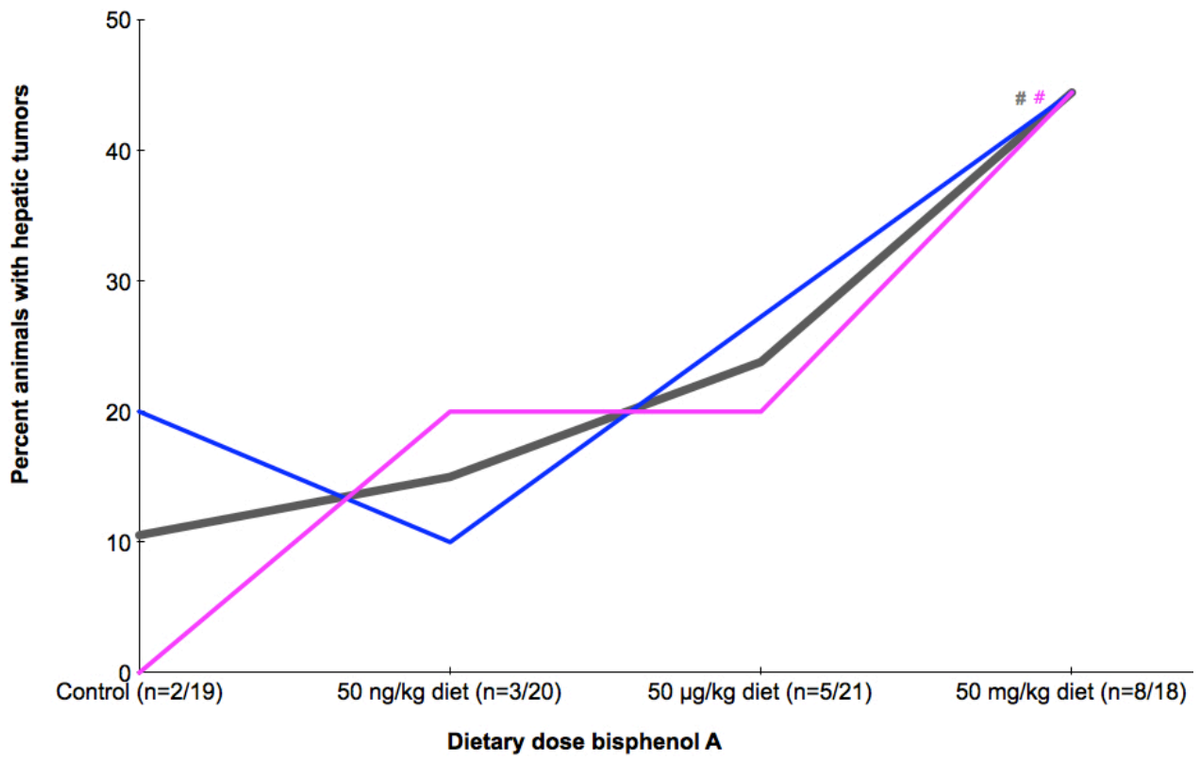
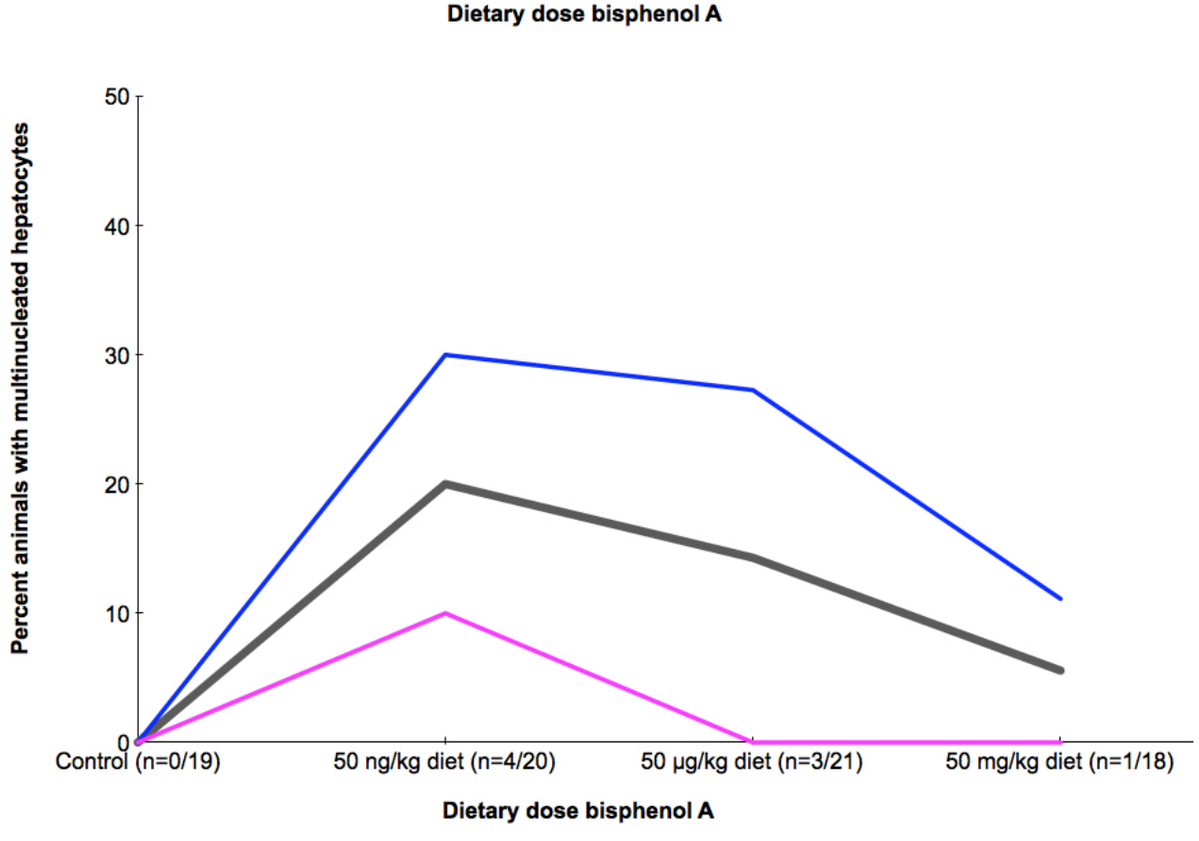


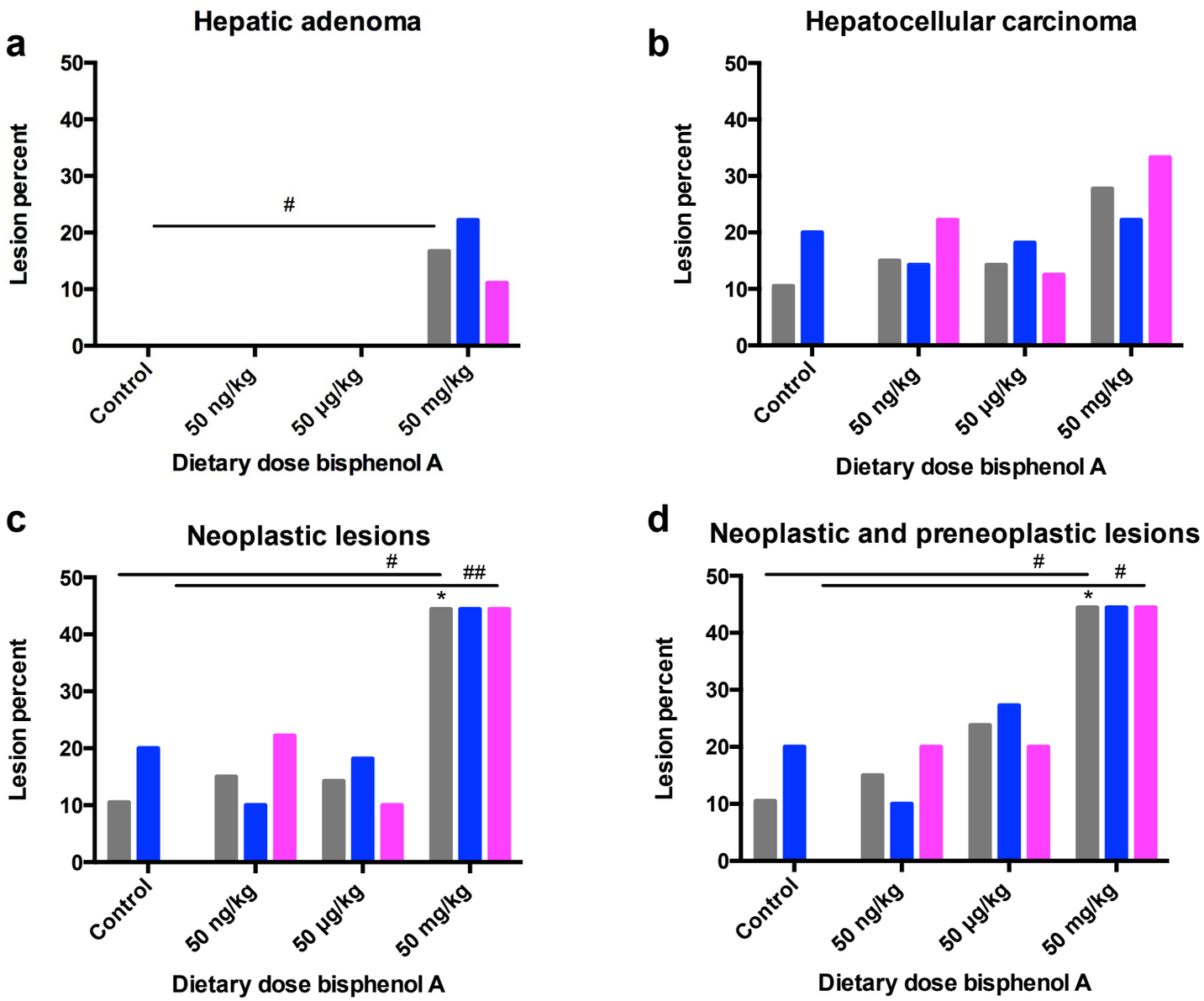
Figure 1.

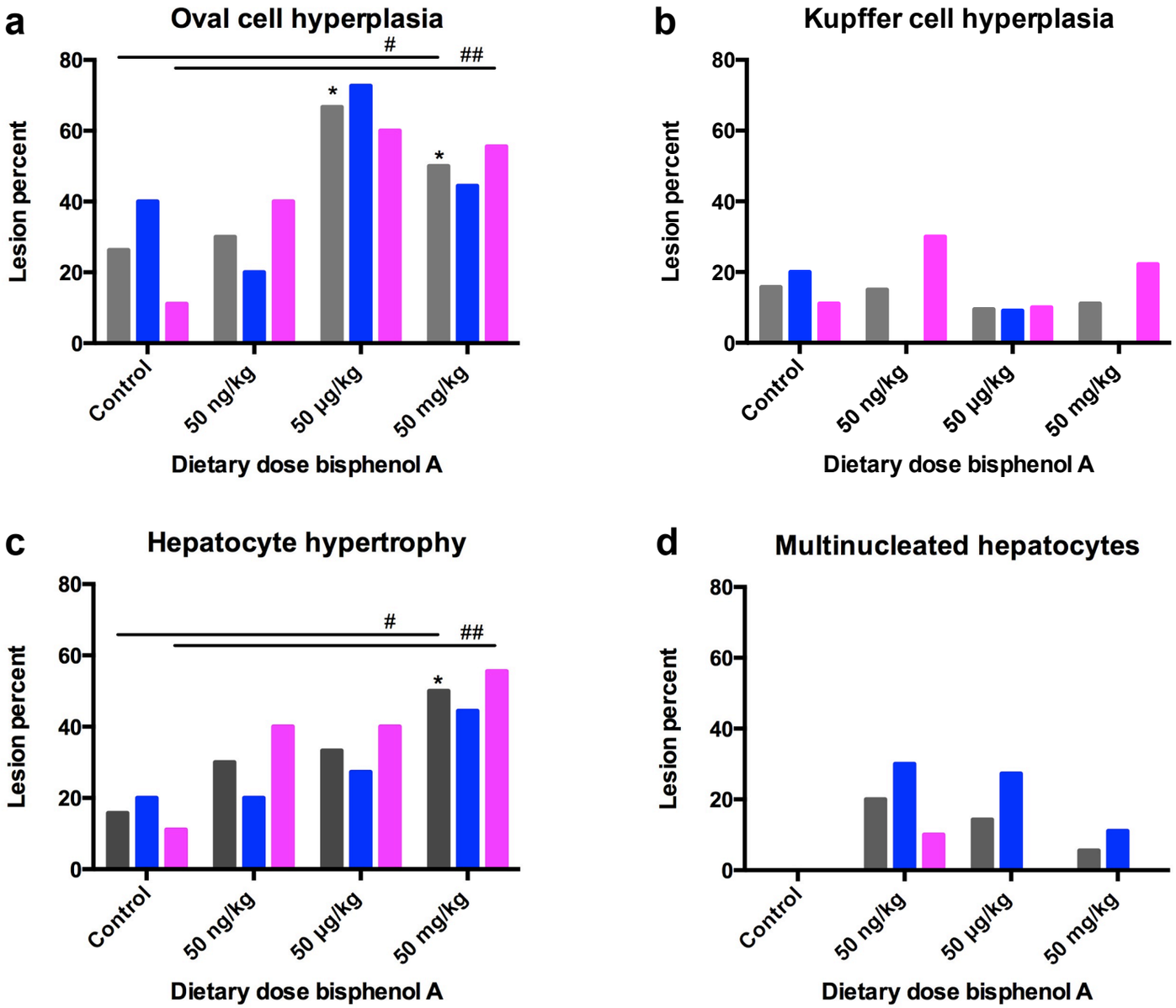
a


Percent animals with hepatic tumors
50
40
30
20
10
0
# #
Control (n=2/19)
50 ng/kg diet (n=3/20)
50 µg/kg diet (n=5/21)
50 mg/kg diet (n=8/18)
Dietary dose bisphenol A

b


Percent animals with multinucleated hepatocytes
50
40
30
20
10
0
Control (n=0/19)
50 ng/kg diet (n=4/20)
50 µg/kg diet (n=3/21)
50 mg/kg diet (n=1/18)
Dietary dose bisphenol A


a
Hepatic adenoma
Lesion percent
0
10
20
30
40
50
#
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A
b
Hepatocellular carcinoma
Lesion percent
0
10
20
30
40
50
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A
c
Neoplastic lesions
Lesion percent
0
10
20
30
40
50
#
##
*
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A
d
Neoplastic and preneoplastic lesions
Lesion percent
0
10
20
30
40
50
#
#
*
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A


a
Oval cell hyperplasia
Lesion percent
80
60
40
20
0
#
##
*
*
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A
b
Kupffer cell hyperplasia
Lesion percent
80
60
40
20
0
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A
c
Hepatocyte hypertrophy
Lesion percent
80
60
40
20
0
#
##
*
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A
d
Multinucleated hepatocytes
Lesion percent
80
60
40
20
0
Control
50 ng/kg
50 µg/kg
50 mg/kg
Dietary dose bisphenol A